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Vapor-Phase Activities of Cinnamon, Thyme, and Oregano Essential Oils and Key Constituents against Foodborne Microorganisms

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The aim of the study presented here was to gain knowledge about the vapor-phase antimicrobial activity of selected essential oils and their major putatively active constituents against a range of foodborne bacterial and fungal strains. In a first step, the vapor-phase antimicrobial activities of three commercially available essential oils (EOs)—cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), and oregano (*Origanum vulgare*)—were evaluated against a wide range of microorganisms, including Gram-negative bacteria (*Escherichia coli*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, and *Salmonella choleraesuis*), Gram-positive bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Enterococcus faecalis*), molds (*Penicillium islandicum* and *Aspergillus flavus*), and a yeast (*Candida albicans*). The minimum inhibitory concentrations (MICs) were generally lower for oregano EO than for the thyme and cinnamon EOs, especially against the relatively resistant Gram-negative. The persistence of the EOs' antimicrobial activities over time was assessed, and changes in the composition of the atmosphere they generated over time were determined using single-drop microextraction (SDME) in combination with gas chromatography−mass spectrometry (GC-MS) and subsequent analysis of the data by principal component analysis (PCA). More relevant chemicals were selected. In addition, the vapor-phase activities of putatively key constituents of the oils were screened against representative Gram-positive (*L. monocytogenes*) and Gram-negative (*S. choleraesuis*) bacteria, a mold (*A. flavus*), and a yeast (*C. albicans*). Of the tested compounds, cinnamaldehyde, thymol, and carvacrol showed the strongest antimicrobial effectiveness, so their MICs, defined as the minimum vapor concentrations that completely inhibited detectable growth of the microorganisms, were calculated. To check for possible interactions between components present in the EOs, cinnamon EO was fortified with cinnamaldehyde and thyme EO with thymol, and then the antimicrobial activities of the fortified oils were compared to those of the respective unfortified EOs using fractional inhibitory concentration (FIC) indices and by plotting inhibition curves as functions of the vapor-phase concentrations. Synergistic effects were detected for cinnamaldehyde on *A. flavus* and for thymol on *L. monocytogenes*, *S. choleraesuis*, and *A. flavus*. In all other cases the fortification had additive effects, except for cinnamaldehyde's activity against *S. choleraesuis*, for which the effect was antagonistic. Finally, various microorganisms were found to cause slight changes over time to the atmospheres generated by all of the EOs (fortified and unfortified) except the fortified cinnamon EO.

KEYWORDS: Vapor-phase antimicrobial effect; essential oils; cinnamaldehyde; carvacrol; thymol; single-drop microextraction

INTRODUCTION

Ideally, commercial techniques for preserving packaged food should maximize its quality and shelf life but have minimal effects on the food. Various methods have been developed or proposed, such as the use of modified atmospheres (MAPs), high pressure, electrical pulses, irradiation, or combinations thereof, but most of them cannot be applied to packaged food (*1*−*5*). A concept that has gained increasing acceptance and interest in recent years is to use natural extracts as antimicrobial agents (*6*−*8*) that could be applied in active packaging (*9*−*11*).

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Most of the relevant studies have only evaluated measures that provide protection when there is direct contact between microorganisms and active agents, which is not the case in most potential applications. However, several works have been published concerning vapor-phase inhibition (*12*−*17*). In a previous paper (*18*), we reported a preliminary evaluation of the activity of four essential oils (EOs; basil, rosemary, clove, and cinnamon) in vapor phase. Promising results were obtained, especially for fungi and Gram-positive bacteria, but the individual chemicals responsible for inhibition were not identified. Furthermore, because different bacteria react differently to bactericides, due to either differences in their inherent characteristics or the development of resistance by adaptation or genetic exchange (*19*), it is clearly important to characterize and compare the atmospheres generated by EOs (and changes in them over time) in the presence of potentially food-degrading bacteria and fungi.

Several techniques have been reported for vapor-phase sampling. Most of them involve solid-phase microextraction (SPME) as a convenient, cheap, and environmentally friendly technique that gives high-quality results in these environments (*20*−*22*). Nevertheless, this technique has some restrictions, such as the limited, although increasing, number of stationary phases available. A technique that has gained acceptance in vapor-phase sampling is single-drop microextraction (SDME) (*23*−*25*). In this technique, a drop (typically 1−3 μL) of an organic solvent is suspended on the tip of a microsyringe as the extraction medium and exposed to the atmosphere under investigation to sample it for a known time at a known temperature. After sampling, the drop is retracted into the syringe and directly injected into a gas chromatograph for analysis. This approach has several advantages compared to SPME, including the larger number of available solvents and the scope it provides to use binary or ternary mixtures. SDME has been previously reported to be a suitable tool for the analysis of the volatile compounds of various EOs (*26*, *27*).

Principal component analysis (PCA) has been widely applied in data mining to investigate the underlying structure and to extract the maximal information from large data matrices. Briefly, in PCA, new orthogonal variables (named latent variables or principal components) are obtained by combining the original ones with the criterion of maximizing the variance of the data. The number of principal components is much lower than the number of original variables, so data handling and visualization are easier. A very interesting feature of the technique is that although PCA greatly reduces the dimensionality of the space, it does not reduce the number of original variables, as it uses all of them to generate the new latent variables. More detailed information about this technique, beyond the scope of this contribution, can be obtained from refs *28* and *29*.

Briefly, the study presented here had several aims. The first was to correlate the vapor-phase antimicrobial activity of three essential oils with the headspace atmosphere they generated using PCA. The next was to select the more relevant chemicals as descriptors to evaluate their individual antimicrobial performance. The final objective was to evaluate potential interactions affecting the antimicrobial activity and to describe the influence of the cultured microorganism on the generated atmospheres.

MATERIALS AND METHODS

Microbial Cultures. The following foodborne microbial strains were selected for their relevance in the food industry: the Gram-positive bacteria *Staphylococcus aureus* (American Type Culture Collection, ATCC 29213), *Bacillus cereus* (Colección Española de Cultivos Tipo, CECT 495), *Enterococcus faecalis* (ATCC 29212), and *Listeria monocytogenes* (ATCC 7644); the Gram-negative bacteria *Escherichia coli* (ATCC 29252), *Yersinia enterocolitica* (CECT 4315), *Salmonella choleraesuis* (CECT 4000), and *Pseudomonas aeruginosa* (ATCC 27853); the yeast *Candida albicans* (ATCC 64550); and the molds *Penicillium islandicum* (CECT 2762NT) and *Aspergillus flavus* (CECT 2687).

The strains were stored at −18 °C in sterilized skimmed milk and subcultured as follows. Gram-positive bacteria were subcultured in Mueller−Hinton agar at 30 °C for 48 h, except for *B. cereus*, which was subcultured in Mueller−Hinton blood agar. Gram-negative bacteria were grown in Mueller−Hinton agar at 30 °C for 24 h. Fungi were subcultured in Sabouroaud cloramphenicol agar either at 30 °C for 48 h (the yeast *C. albicans*) or at 36.5 °C for 7 days (the molds *P. islandicum* and *A. flavus*).

Essential Oils and Chemicals. The essential oils were supplied by Artibal (Sabiñánigo, Spain). Oils from the following plant species were tested in this work: *Cinnamomum zeylanicum* [cinnamon, Chemical Abstracts Service (CAS) Registry No. 8015-91-6], *Thymus vulgaris* (thyme, CAS Registry No. 8015-73-4), and *Origanum vulgaris* (oregano, CAS Registry No. 8007-11-2).

The chemicals used in the analyses of the antimicrobial activity of specific compounds and the atmospheres were the following: α-pinene (98%, CAS Registry No. 80-56-8), camphene (95%, CAS Registry No. 79-92-5), β-pinene (99%, CAS Registry No. 1872-67-3), *p*-cymene (99%, CAS Registry No. 99-87-6), (+)-limonene (97%, CAS Registry No. 5989-27-5), 1,8-cineole (99%, CAS Registry No. 470-82-6), linalool (2,6-dimethylocta-2,7-dien-6-ol, 97%, CAS Registry No. 78-70-6), camphor (96%, CAS Registry No. 76-22-2), (−)-borneol [1,7,7-trimethyl-(1*S*)-*endo*-bicyclo[2.2.1]heptan-2-ol, 98%, CAS Registry No. 464-45-9], estragol [1-methoxy-4-(2-propenyl)benzene, 98%, CAS Registry No. 140-67-0], *trans*-cinnamaldehyde (99%, CAS Registry No. 14371-10-9), eugenol [2-methoxy-4-(2-propenyl)phenol, 99%, CAS Registry No. 97-53-0], and α-humulene (98%, CAS Registry No. 6753-98-6) supplied by Aldrich (Sigma-Aldrich Química S.A., Madrid, Spain); thymol [5-methyl-2-(1-methylethyl)phenol, >99.5%, CAS Registry No. 89-83-8] and β-caryophyllene (99%, CAS Registry No. 87-44-5) by Sigma (Sigma-Aldrich Química S.A., Madrid, Spain); and α-terpinolene [1-methyl-4-(1-methylethylidene)cyclohexene, >97%, CAS Registry No. 586-62-9], hydrocinnamaldehyde (3-phenylpropionaldehyde, >90%, CAS Registry No. 104-53-09), carvacrol (5-isopropyl-2-methylphenol, >97%, CAS Registry No. 499-75-2), verbenone [4,6,6-trimethylbicyclo(3,1,1)hept-3-en-2-one, CAS Registry No. 18309-32-5, >97%], and *p*-xylene (CAS Registry No. 106-42-3, >98%,) by Fluka (Sigma-Aldrich Química S.A., Madrid, Spain).

Antimicrobial Activity Tests. The modified vapor diffusion test used in this study has already been described in detail in ref *18*. Briefly, the solidified agar medium was inoculated with 100 μL of a physiological saline solution containing 10^5 colony-forming units (CFUs) of the microorganism under study. Then, 10 μL of each ethyl ether (GC quality, Merck, Darmstadt, Germany) dilution of the antimicrobial agent (EO or specific chemical) was added to a 10-mm sterile blank filter and placed on the medium-free cover of the dish. The Petri dish was then sealed using sterile adhesive tape (Deltalab, Rubi, Spain). Blanks (containing 10 μL of diethyl ether) were triplicated and included in every experimental set.

After the incubation period, the minimal inhibitory concentration (MIC, expressed as microliters of EO per liter of atmosphere above the agar surface or per decimeter squared of agar surface) was calculated. MICs can be calculated and expressed in several ways (*27*), but in this contribution our definition is the minimum vapor concentration of an active principle that totally inhibits the test microorganism's growth (i.e., results in no detectable bacterial or fungal growth) by comparison with control tests because total inhibition is not a relative measure. All tests were performed in triplicate.

SDME. Before each extraction, the syringe was rinsed with the organic solvents acetone, ethanol, *p*-xylene, in sequence, 10−15 times each, to avoid formation of air bubbles and carry-over of compounds. Then, 3 μL of *p*-xylene containing 6.7 μg/g of verbenone, used as the injection standard, was drawn into the syringe [Hamilton 85RN (26S/

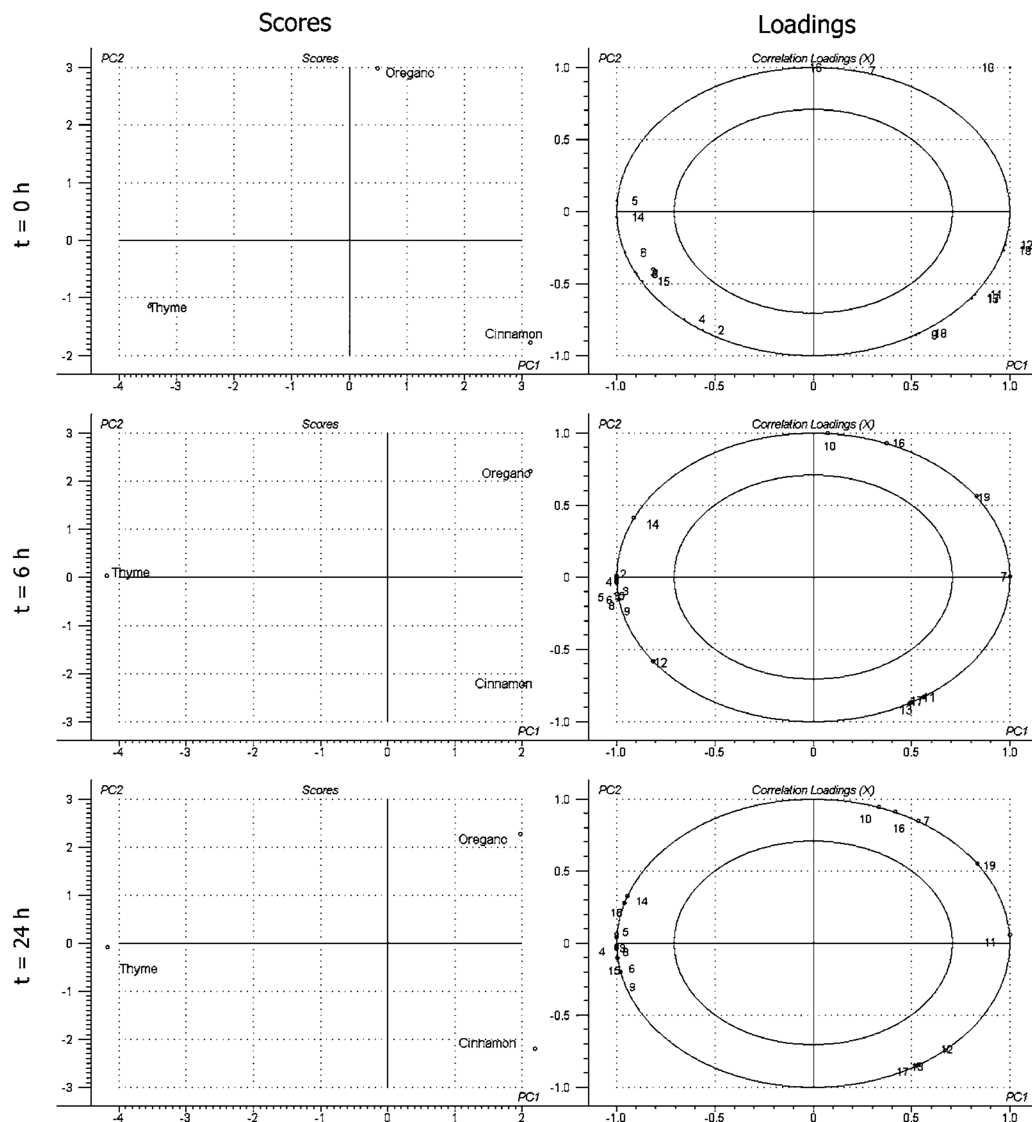


Figure 1. Principal component analysis of the atmosphere generated by oregano, thyme, and cinnamon essential oils. Peaks: 2, α-pinene; 3, camphene; 4, β-pinene; 5, *p*-cymene; 6, limonene; 7, 1,8-cineole; 8, α-terpinolene; 9, linalool; 10, camphor; 11, borneol; 12, estragol; 13, cinnamaldehyde; 14, thymol; 15, bornyl acetate; 16, carvacrol; 17, eugenol; 18, β-caryophyllene; 19, α-humulene. Panels: top, test time = 0 h; middle, test time = 6 h; bottom, test time = 24 h.

51 mm/needle type 2; Hamilton Bonaduz AG, Bonaduz, Switzerland] and suspended over the vial (calibration samples) or the Petri dish (test samples) using a metal stand. The needle was introduced to a given depth, and the plunger was depressed to expel 2.5 μL of the solvent. The drop formed was discarded, and the plunger was then fully depressed to generate a 2.5 μL droplet of solvent on the needle tip. When the extraction time (5 min) was complete, the drop was retracted, the syringe was taken out of the vial, and the solvent was injected into the GC.

For calibration (i.e., partition constant, K_5, calculations) vapor phases containing different concentrations of the analytes were generated by evaporating a known volume (5 μL) of diethyl ether solutions of the chemicals under study in 20 mL of silanized glass vials (Teknocroma, Barcelona, Spain). The vials were then thermostated at 80 °C for 4 min to evaporate the solution and generate an atmosphere that was sampled at 30 °C as described above. A calibration graph was constructed by plotting the ratio of the signals obtained from the analyte and the injection standard against the concentration ratio of the two chemicals in the drop to correct for small drop volume variations. The partition coefficient after 5 min of extraction (K_5, the extraction time used when sampling the atmospheres inside the Petri dishes) was calculated for each analyte from the slope of the common graph.

To analyze atmospheres in the Petri dishes, they were sampled as follows. First, a sampling port was made by drilling a 0.7 mm hole at

Table 1. Antimicrobial Activity of the Atmospheres Generated by the Pure Essential Oils against the Selected Foodborne Pathogens[a]

	cinnamon		oregano		thyme	
microorganism	μL/L	μL/dm^2	μL/L	μL/dm^2	μL/L	μL/dm^2
Gram-positive						
S. aureus	34.9	3.1	13.1	1.2	87.3	7.9
E. faecalis	52.4	4.7	34.9	3.2	87.3	7.9
L. monocytogenes	34.9	3.1	26.2	2.4	175	15.7
B. cereus	17.5	1.6	17.5	1.6	131	11.8
Gram-negative						
E. coli	17.5	1.6	13.1	1.2	52.4	4.7
P. aeruginosa			175	15.7		
Y. enterocolitica	17.5	1.6	4.4	0.4	34.9	3.1
S. choleraesuis	131	11.8	17.5	1.6	131	11.8
mold						
A. flavus	13.1	1.2	17.5	1.6	175	15.7
P. islandicum	8.7	0.9	17.5	1.6	34.9	3.1
yeast						
C. albicans	13.1	1.2	17.5	1.6	26.2	2.4

[a] Results are shown as minimum inhibitory concentrations (expressed as μL of EO added per L of headspace or dm^2 of agar surface).

Table 2. Adimensional Partition Coefficients for 5 min Sampling Time (K_5) as the Ratio between Concentration Generated in the Headspace and the Concentration Found in the Drop, Obtained by GC-MS Determination ($n = 3$, RSD < 15%)

chemical	$K_5 \times 10^3$	chemical	$K_5 \times 10^3$
α-pinene	4.01 ± 0.55	borneol	5.32 ± 0.57
camphene	3.90 ± 0.44	estragol	3.15 ± 0.20
β-pinene	3.58 ± 0.32	cinnamaldehyde	4.31 ± 0.11
p-cymene	3.16 ± 0.47	thymol	10.6 ± 0.07
limonene	3.12 ± 0.36	carvacrol	13.1 ± 0.16
1,8-cineole	3.97 ± 0.44	bornyl acetate	4.59 ± 0.23
α-terpinolene	10.9 ± 0.15	eugenol	58.7 ± 9.20
linalool	6.66 ± 0.20	β-caryophyllene	4.26 ± 0.21
camphor	3.36 ± 0.29	α-humulene	4.81 ± 0.17

Table 3. Antimicrobial Effect of the Individual Chemicals[a]

	microorganism			
chemical	*S. choleraesuis*	*L. monocytogenes*	*A. flavus*	*C. albicans*
eugenol	20 (28)	0 (8)	52 (56)	57 (69)
cinnamaldehyde	39 (42)	34 (37)	100	91 (94)
hydrocinnamaldehyde	39 (56)	0 (6)	11 (13)	91 (97)
thymol	56 (67)	80 (84)	67 (72)	100
carvacrol	46 (50)	58 (61)	89 (100)	100
camphor	0	0	0	0 (19)
estragol	0	0	0	0
1,8-cineole	0	0	0	0
p-cymene	0	0	0	0
β-caryophyllene	0	0	0	0
limonene	0	0	0	0
linalool	10 (13)	0 (28)	0	5 (67)

[a] Results expressed as percentage of inhibition. Roman numbers represent total inhibition (no visible growth of colonies), whereas italic numbers account for the delay effect (colonies significantly smaller or less numerous compared to control tests). 100 represents total inhibition; 0 represents no inhibition.

the side of each dish approximately 6 mm from the bottom and covered using an adhesive Teflon-faced septum (Análisis Vínicos, Tomelloso, Spain). Then, the microorganism was introduced and cultured as described previously (*18*). At predetermined times, the syringe was introduced through the septum and SDME was conducted as described above.

Gas Chromatography−Mass Spectrometric (GC-MS) Analysis. GC-MS analyses were performed using a Hewlett-Packard 6890 gas chromatograph (Wilmington, DE) equipped with a 5973 mass selective detector and an A HP-5 MS (60 m × 0.25 mm, 0.25 μm film thickness) capillary column.

The temperature program for the gas chromatography was as follows: initial temperature, 75 °C, raised by 10 °C/min to 190 °C, then by 20 °C/min to 280 °C, which was held for 5 min. The injector temperature was 270 °C, injection was in splitless mode (splitless time = 18 s), and the temperature of the transfer line was 280 °C. The carrier gas was helium (99.999% purity, 1.0 mL/min) supplied by Carburos Metálicos (Barcelona, Spain). Screening of the chromatograms was performed in scan mode, from m/z 45 to 250, at a rate of 6.61 uma/s. The detected compounds of interest were then quantified by selected ion monitoring (SIM) analysis, once their characteristic masses had been selected from their full spectra.

PCA. The starting data matrix is formed by $n = 5$ cases (independent replicates for each individual experiment, that is, deployment time and/or presence/absence of microorganism) and $p = 19$ variables (defined as the peak area obtained for each individual component). Data were standardized by autoscaling prior to statistical analysis (subtraction of the mean and division of the variance). Statistical analyses were performed by means of the Unscrambler software package, version 9.1 (CAMO Software AS, Trondheim, Norway).

RESULTS AND DISCUSSION

Table 1 shows the antimicrobial activity (expressed as MIC, defined as the minimum concentration in the headspace that leads to non detectable growth of the microorganism) found for cinnamon, oregano, and thyme commercial essential oils against the selected array of microorganisms. As can be seen, thyme was highly ineffective in this study, unlike others in which it has been found to be one of the most effective EOs (*30*, *31*); oregano EO was the most inhibitory overall against bacteria, and cinnamon EO was the most effective against fungal strains. The next step in the investigation was to correlate these results with variations in the composition of the atmospheres generated by the essential oils in the Petri dishes using PCA.

To quantify the volatiles inside the Petri dishes to carry out PCAs, SDME was used as described under Materials and Methods. Calibration was performed by calculating the partition coefficients (which theoretically depend only on the extraction temperature and sampling time) for the analytes after 5 min of sampling (K_5), again as described under Materials and Methods. Partition coefficients are dimensionless and can be calculated using the following simple equation (eq 1)

$$K_5 = \frac{C_{h,5}}{C_{o,5}} \quad (1)$$

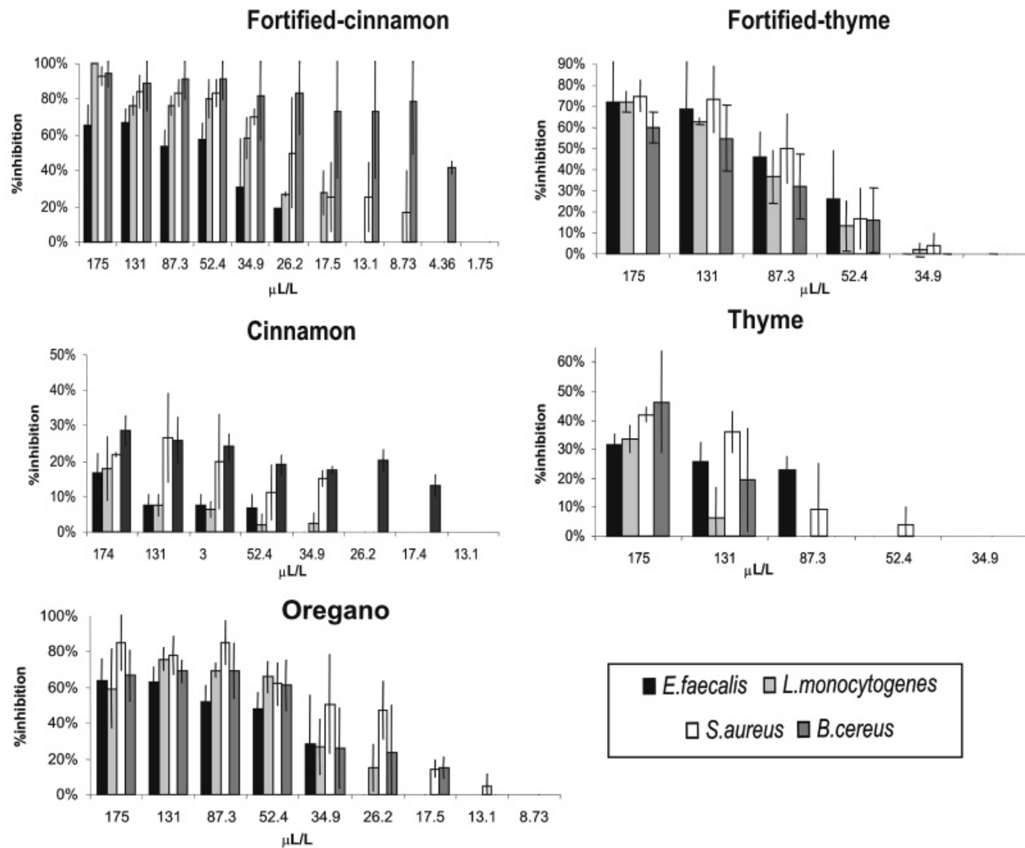
where $C_{h,5}$ is the concentration of the analyte in the headspace in μg/L of headspace and $C_{o,5}$ is the concentration in the drop as μg of chemical/L of solvent measured by GC-MS after 5 min of sampling. Results are presented in **Table 2**. Of course, the composition of the headspace atmosphere, that is, the presence/absence and abundance of other chemicals in the headspace, could affect the extraction of an individual component. Therefore, the possibility that such interactions may have affected the results was checked by calculating K_5 values for several single-component atmospheres (α-humulene, thymol, and *p*-cymene). No differences ($p < 0.05$) were found with those reported in the table, indicating that such interactions had little or no effect on the results. The concentrations of analytes in samples of atmospheres in the Petri dishes, which were obtained by sampling at 30 °C for 5 min, were also calculated using eq 1.

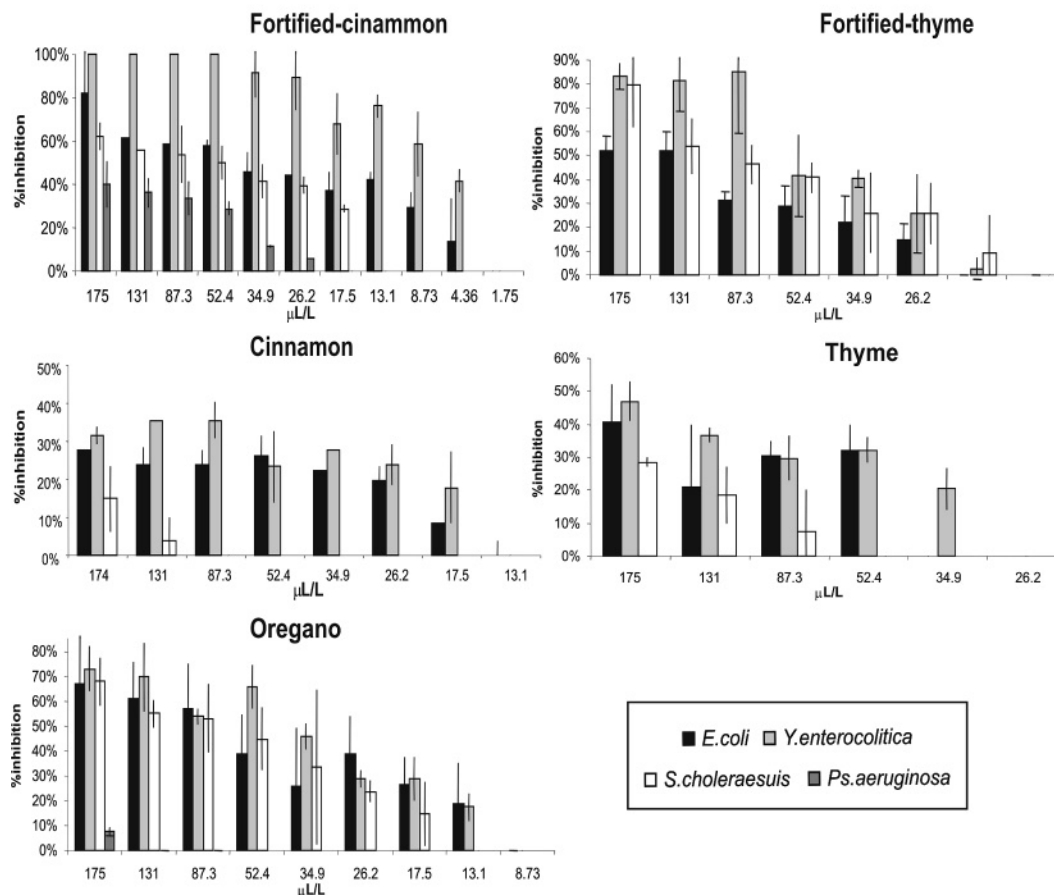
In a previous contribution, the bactericidal effect of cinnamon EO was described (*18*). There, it was shown that the compounds responsible for the inhibition need to be present in the atmosphere to get the effect on the lag phase of the microorganism (*32*−*34*). Thus, samples were taken ($n = 5$) from Petri

Table 4. MICs of the Individual Constituents[a]

	L. monocytogenes		*S. choleraesuis*		*A. flavus*		*C. albicans*	
	μL/L	μL/dm²	μL/L	μL/dm²	μL/L	μL/dm²	μL/L	μL/dm²
cinnamaldehyde	21.8	2.0	4.4	0.4	21.8	2.0	4.4	0.4
carvacrol	21.8	2.0	10.9	1.0	21.8	2.0	10.9	1.0
thymol	21.8	2.0	10.9	1.0	43.6	3.9	10.9	1.0

[a] Results expressed as μL of active compound added per L of headspace or dm² of agar surface.


Gram +
Fortified-cinnamon
Fortified-thyme
Cinnamon
Thyme
Oregano
%inhibition
μL/L
E.faecalis
L.monocytogenes
S.aureus
B.cereus


Gram -
Fortified-cinammon
Fortified-thyme
Cinnamon
Thyme
Oregano
%inhibition
μL/L
E.coli
Y.enterocolitica
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Ps.aeruginosa

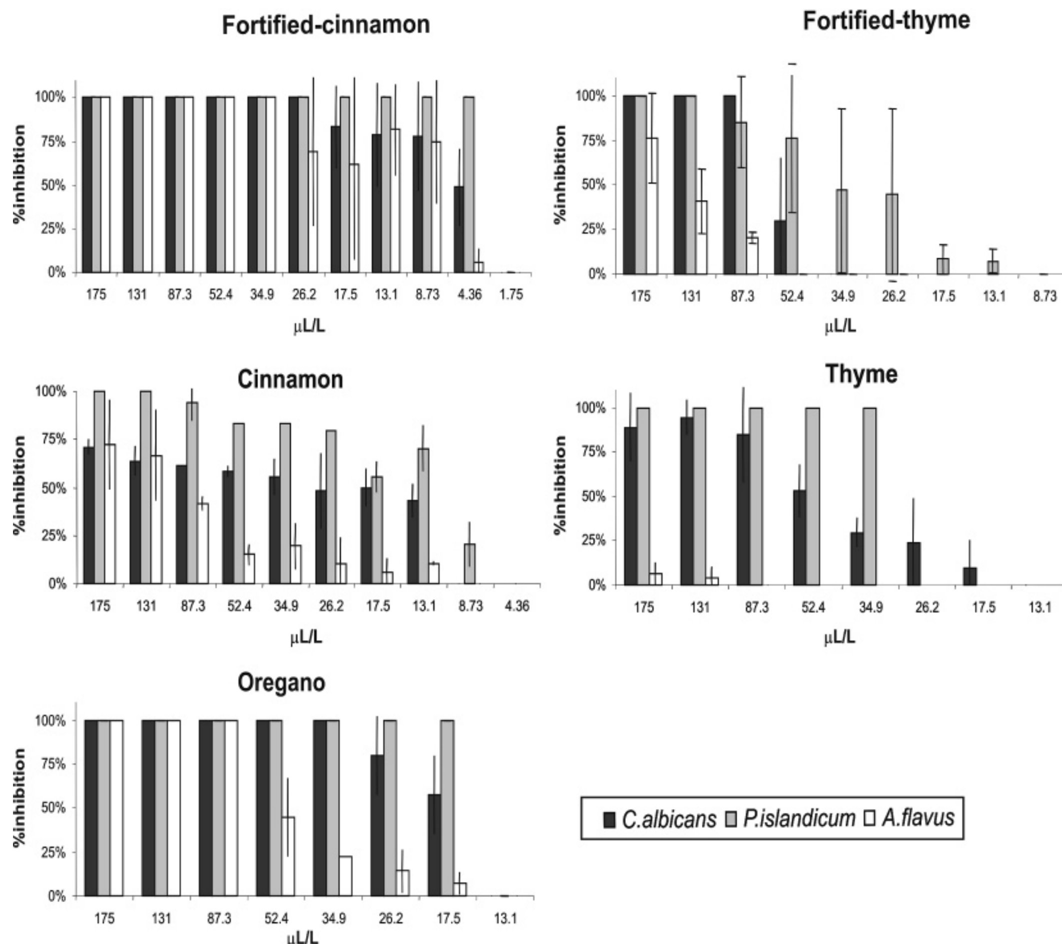

Figure 2. Effects of the fortified and unfortified EOs in the vapor phase on the test microorganisms as a function of concentration, milligrams per liter ($n = 3$). *Y* error bars, standard deviation; % inhibition $= T/C \times 100$; *T*, inhibition diameter; *C*, diameter of no inhibition (90 mm).

Table 5. Fractional Inhibitory Concentration (FIC) Indices for Cinnamaldehyde-Fortified Cinnamon EO and Thymol-Fortified Thyme EO[a]

	FIC cinnamaldehyde	FIC thymol
L. monocytogenes	0.7	**0.5**
S. choleraesuis	3.6	**0.3**
A. flavus	**0.1**	**0.4**
C. albicans	0.9	1.2

[a] Bold values represent synergy.

dishes with each EO at various times during the incubations (see Materials and Methods for a description of the testing procedure), and the correlations between the composition of the oils and their inhibitory effects on the screening organisms were explored using PCA. The results obtained are shown in **Figure 1** for samples taken immediately after the Petri dish had been closed ($t = 0$; top panels), after 6 h ($t = 6$; middle panels), and 24 h ($t = 24$; bottom panels).

The scores, which illustrate the relationship (positive or negative) of each EO as a whole with the different principal components (PC), can be used to objectively compare the atmospheres generated. As can be seen in the figure scores clearly separate the three types of EO. PC1 discriminates thyme EO, whereas PC2 separated oregano EO from cinnamon EO. It is also relevant that the scores graph changes slightly from 0 to 6 h and remains unchanged afterward, as demonstrated also in the loadings graph.

Loadings, which can be defined roughly as the correlations between the individual variables (i.e., individual chemicals) and the principal components defined (i.e., atmosphere generated), demonstrate that different chemicals were found as the more relevant as a function of both the EO and the incubation time. For samples taken at $t = 0$, two PCs were needed to explain 100% of the variance (PC1, 62%; PC2, 38%). The main explanatory variables were carvacrol and 1,8-cineole for oregano EO; borneol, eugenol, cinnamaldehyde, β-caryophyllene, and linalool for cinnamon EO; and α-pinene, β-pinene, camphene, limonene, and bornyl acetate for thyme EO. For the atmospheres generated after 6 h, the variance in activity of the oregano, cinnamon, and thyme EOs was fully described by two PCs (PC1, 73%; PC2, 27%), and the explanatory variables were carvacrol, camphor, and α-humulene for the oregano EO; borneol, eugenol, and cinnamaldehyde for the cinnamon EO; and α- and β-pinene, camphene, *p*-cymene, limonene, α-terpinolene, β-caryophyllene, and bornyl acetate for the thyme EO. Finally, after 24 h (PC1, 72%; PC2, 28%) carvacrol, 1,8-cineole, camphor, and α-humulene were the main explanatory variables for the oregano EO; estragol, eugenol, and cinnamaldehyde were those for the cinnamon EO; and α- and β-pinene, camphene, *p*-cymene, limonene, α-terpinolene, and bornyl acetate were those for the thyme EO.

Thus, the individual effects of the following chemicals were examined: carvacrol, camphor, and 1,8-cineole as important explanatory variables for oregano EO; eugenol, estragol, linalool,

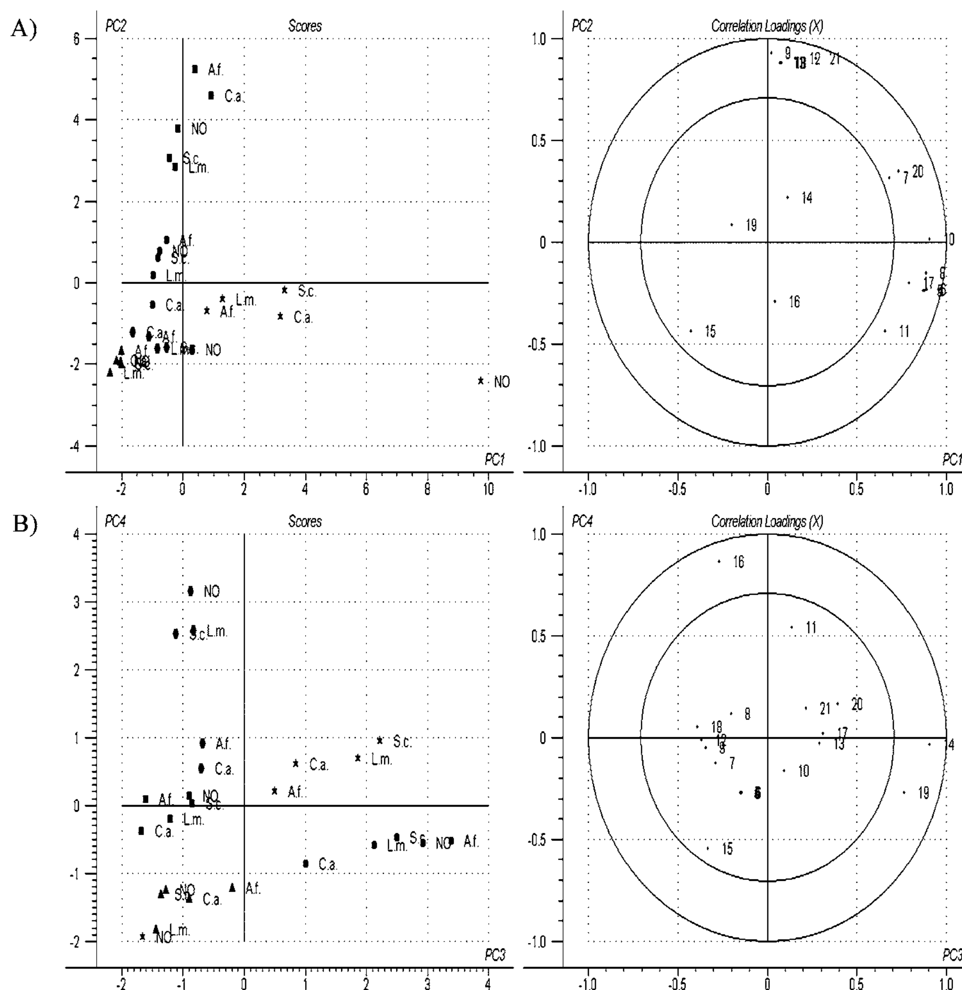

Figure 3. Principal component analysis of the atmosphere generated by cinnamon-fortified, thyme-fortified, oregano, thyme, and cinnamon essential oils (sampling time = 24 h): (**A**) scores (●, cinnamon; ■, oregano; ⬢, fortified-thyme; ★, thyme; ▲, fortified-cinnamon; NO, no microorganism; *S.c*, *Salmonella choleraesuis*; *L.m*, *Listeria monocytogenes*; *C.a*, *Candica albicans*; *A.f*, *Aspergillus flavus*); (**B**) loadings (3, α-pinene; 4, camphene; 5, β-pinene; 6, *p*-cymene; 7, limonene; 8, 1,8-cineole; 9, α-terpinolene; 10, linalool; 11, camphor; 12, borneol; 13, estragol; 14, cinnamaldehyde; 15, thymol; 16, bornyl acetate; 17, carvacrol; 18, eugenol; 19, β-caryophyllene; 20, α-humulene).

and cinnamaldehyde for cinnamon EO; and limonene, *p*-cymene, and β-caryophyllene for thyme EO. Interestingly, thymol was not an important explanatory variable for the thyme EO used (see **Figure 1**), so we hypothesize that the poor activity of this EO is mainly due to its low content of this chemical, because it is reportedly responsible for most of the EO's antimicrobial activity (*31*, *35*). To fully test this hypothesis, thymol was included in the individual study.

Table 3 shows the antimicrobial activities of the selected chemicals. Hydrocinnamaldehyde was also added to the test set to check the possible importance of minor structural differences (double-bond hydrogenation compared to cinnamaldehyde) for the inhibitory activity. Eugenol, which has been reported to have antimicrobial activity in direct contact (*36*), gave poor inhibitory results in vapor phase, being effective against only *C. albicans* and to a minor extent against *S. choleraesuis* and *A. flavus*. Better results were obtained for thymol and carvacrol, which displayed vapor-phase inhibition of fungi and also significant inhibition against both Gram-negative and Gram-positive bacteria. Thymol and carvacrol gave similar results for *C. albicans*, *A. flavus*, and *L. monocytogenes*, but thymol was significantly ($p < 0.05$) more effective than carvacrol against *S. choleraesuis*. These findings clearly indicate that vapor-phase inhibition by oregano EO could be largely attributed to carvacrol, but eugenol does not appear to play a major role in the antimicrobial activity of cinnamon EO. The position of the hydroxyl group seems to influence the components' activity against Gram-negative bacteria, as demonstrated by the difference in activity between carvacrol and thymol against *S. choleraesuis*.

Furthermore, the significance of the phenolic ring was demonstrated by the lack of activity of the monoterpene cyclic hydrocarbon *p*-cymene, which is a precursor of carvacrol and thymol. Cinnamaldehyde and hydrocinnamaldehyde had very similar inhibitory effects against *C. albicans*, but cinnamaldehyde was much more inhibitory than hydrocinnamaldehyde against *S. choleraesuis* and (even more so) against *A. flavus* and *L. monocytogenes*.

Therefore, cinnamaldehyde, thymol, and carvacrol were the most effective chemicals in accordance with results presented by Valero and Giners (*37*) and were selected for further experiments. No major health concerns are involved, because they are included in the A-list published by the Council of Europe and classified as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (*38*, *39*). MICs of the pure chemicals against these microorganisms were calculated and are shown in **Table 4**. No differences among the three chemicals tested were found in terms of their MICs for *L. monocytogenes* (21.8 μL/L of headspace), but for *S. choleraesuis* and *C. albicans*, thymol and carvacrol were less inhibitory (MIC = 10.9 μL/L in both cases) than cinnamaldehyde (4 μL/L),

although these differences were not statistically significant. For *A. flavus*, carvacrol and cinnamaldehyde were more strongly inhibitory (21.8 μL/L) than thymol (43.6 μL/L).

It is interesting to compare the inhibitory effects of the individual chemicals to their effects as constituents of the EOs. Various techniques have been proposed for exploring such relationships, but we chose to use fractional inhibitory concentration (FIC) indices (*40*, *41*), calculated as the ratio between the MICs obtained for the compounds as parts of a mixture and the MICs for the pure compounds. FIC values of $\leq$0.5, 0.5−4, and >4 indicate synergistic effects, additive effects, and antagonistic effects, respectively.

Two mixtures were evaluated: cinnamaldehyde-fortified cinnamon essential oil and thymol-fortified thyme essential oil. Results are shown in **Table 5**. As can be seen, cinnamaldehyde only had synergistic effects with cinnamon EO for inhibiting *A. flavus*, and additive effects were found for the other organisms, verging on antagonistic for *S. choleraesuis*. Different trends were found for thymol and thyme EO: they had synergistic effects on *L. monocytogenes*, *S. choleraesuis*, and *A. flavus* and an additive effect on *C. albicans*. These results are fully consistent with those shown in **Table 4** and demonstrate once again that the antimicrobial activity of thyme EO is heavily dependent on its thymol concentration.

The vapor-phase inhibition curves obtained for oregano EO and both fortified and unfortified thyme and cinnamon EOs were then calculated and are shown in **Figure 2**. Both fortified EOs demonstrated much higher activity than the respective commercial, unfortified EOs. Although cinnamon EO has lower MICs than oregano EO for fungi, the inhibition curves (**Figure 2**) show that oregano EO was more inhibitory at concentrations between 175 and 17.5 μL of EO/L. Oregano EO was more effective than fortified thyme and equally effective for fungal inhibition as fortified cinnamon EO, whereas the latter was the most effective against bacteria, especially for *Y. enterocolitica* and *P. aeruginosa* (Gram-negative) and *B. cereus* (Gram-positive). We find the increased antimicrobial activity of cinnamaldehyde-fortified cinnamon EO against Gram-negative bacteria, including the most resistant *P. aeruginosa*, to be highly interesting, as well as its activity against fungi (because it totally inhibits the growth of *P. islandicum*, *A. flavus*, and *C. albicans* at concentrations of 4.36, 34.9, and 26.2 μL/L of EO, respectively). These activities are significantly higher than those we found in our previous study (*18*), where the vapor-phase activities of all of the EOs against Gram-negative bacteria were significantly lower (and no inhibition of *P. aeruginosa* was detected).

Finally, the influence of the cultured microorganism on the composition of the generated atmospheres over time was analyzed, and the results are presented in **Figure 3** ($t = 6$ h). As can be seen, 76% of the variance can be explained by three principal components. PC1 describes variation of the atmosphere generated by thyme EO attributable to the microorganisms. The amounts of α- and β-pinene, camphene, and *p*-cymene in the atmosphere were maximal (and significantly different from those in blank samples) in the presence of *A. flavus* and *S. choleraesuis*. PC2 distinguishes cinnamon and oregano EO from the fortified EO, which are defined by thymol (*16*) and cinnamaldehyde (*15*). The fortified cinnamon atmosphere was not affected by the microorganisms, and differences in the fortified thyme atmosphere were detected only in the presence of *C. albicans*. PC3 differentiates the effects of the diverse microorganisms on the atmospheres of both the cinnamon and oregano EOs. *L. monocytogenes* and *C. albicans* induced significant differences in the detected amounts of carvacrol, thymol, borneol, camphor, and linalool in the oregano EO atmosphere. In addition, there were significant differences in the eugenol contents of atmospheres generated by cinnamon EO in the presence of *C. albicans* and *L. monocytogenes* and between blank samples and samples with *A. flavus* cultures.

Thus, the presence of different microorganisms proved to have a clear influence on the atmospheres generated by the different antimicrobial solutions. These differences could be attributed to diverse interactions and/or biotransformation routes and provide a possible basis to elucidate the mechanisms involved in the antimicrobial activity of this potential food preservation technique. However, the main tasks for the near future are to develop and evaluate active antimicrobial packaging systems based on these antimicrobial substances.

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